AN IMPROVED SUPPORT FOR CHROMOPHORIC ELEMENTS

The present invention relates to improving the detection of chromophoric or chromogenic signals directly or indirectly connected to chemical or biological cells,

5 molecules or macromolecules fixed to supports.

The term "chromophoric signal" or "chromogenic signal" means any light signal emitted directly or indirectly after excitation by suitable light or after enzymatic transformation of a chromogenic substrate. The

10 category of chromophoric or chromogenic signals includes colorimetric, photoluminescent, fluorescent, chemoluminescent and bioluminescent signals and the like. Such signals are emitted either directly by the molecules of interest or by elements added to them and/or grafted

15 onto them, such as colorant molecules or certain types of semiconducting nanostructures such as quantum wires or boxes.

The technologies used in cell and molecular biology to detect and quantify the presence of a molecule usually

20 exploit such signals and are detected by spectrometers, fluorimeters or luminometers equipped with photomultipliers.

The term "molecule" as used here means any type of molecule or macromolecule, isolated or bonded to a

25 structure or to another molecule. Examples that can be cited are nucleic acids or oligonucleotides, proteins or polypeptide sequences and antibodies or fragments thereof. The term "molecule" can also mean chemical molecules with a particular affinity for a specific

30 ligand. Examples that can be mentioned are the molecules obtained from high throughput syntheses and their selection using combination chemistry techniques.

The accessibility of biological material and the search for the presence of a particular molecule in a

35 complex mixture have led to the development of miniaturized techniques that employ "chips", "microarrays" and "macroarrays". The molecules to be

investigated or to be quantified (targets) are brought into the presence of specific known ligands (probes) fixed on a support or a substrate that is currently formed from glass, silica, nylon or a metal. A target is

5 detected when it couples to a fixed probe (or specific ligand) because the target carries a chromophore (it is possible for the probe to carry the chromophore).

The term "support" or "substrate" as used here means any means that can support chromophoric elements. As a

10 result, it may be a monolayer structure or a multilayer structure. The monolayer or one of the layers of the multilayer can be a substrate that is commercially available and possibly modified.

The respective arrangements of targets, probes and

15 chromophores are interchangeable. This text refers to probes integral with a support and with which the targets interact, but it is possible for the targets to be integral with the support, and the probes could then interact therewith.

20 The probes can be fixed on their supports in different manners. Examples are chemical addressing, electrochemical addressing, or addressing using a technology similar to the inkjet technology used in printers.

25 A detailed review of that subject can be found in the article by F. Bertucci et al., Human Molecular Genetics (1999), $\underline{8}$, n° 9, pp. 1715-1722.

The sensitivity of the detection means currently used necessitates the presence of at least 10^4 molecules

30 of a single category to obtain a significant response. Further, as more and more probes are being mounted on ever smaller supports, it is becoming particularly difficult to detect the origin of luminescent signals or colorimetric signals generated after coupling or

35 probe/target hybridization.

Conventional solutions for improving measurement sensitivity have up to now consisted of enhancing either

the emitted signal, for example using radioactive targets, or the quantity of material that can be hybridized or coupled. However, such solutions are not satisfactory.

5   Other solutions require optical systems with a very wide numerical aperture and/or immersed systems, which are incompatible with the rapid scanning requirements of large arrays.

United States patent US-A-6 008 892 describes a

10   device for supporting chromophoric elements which comprises a rigid base with a reflective surface, for example metal, covered with a transparent layer, for example silica, on which the chromophoric elements are fixed. The optical pathway between the reflective

15   surface of the base and the upper face of the transparent layer is an odd multiple of one quarter wavelength of the excitation light for the chromophoric elements, such that the standing wave of the incident excitation light has an antinode at the level of the chromophoric elements.

20   Chromophore excitation is then a maximum. The reflective base can also enhance collection of the fluorescence emitted in response to excitation, by practically doubling the solid angle over which fluorescence is collected by a microscope system.

25   The aim of the present invention is to further improve detection of the light emitted by chromophoric elements in response to light excitation.

To this end, the invention provides a device for supporting chromophoric elements of the type defined

30   above, which can concentrate at least a portion of the light emitted by the chromophoric elements in a selected region of space, to allow it to be captured. The term "concentrate" as used here means firstly, recovering the light emitted by the chromophoric elements in a portion

35   of the region around it, then constraining that recovered light to reach one or more collection zones.

Thus, the invention provides a device for supporting at least one chromophoric element, comprising a substrate having an upper surface on which said chromophoric element is fixed and means for enhancing the quantity of light emitted by the chromophoric element towards a collecting device, said means forming part of a group comprising reflective means placed in the substrate at a distance from its upper surface, characterized in that this group also comprises:

- microlenses each associated with a chromophoric element and functioning in transmission or in reflection;
- diffraction means placed at a distance from the chromophoric element or elements and functioning in transmission or in reflection;
- first reflective means and second reflective means parallel to each other and placed either side of the chromophoric element or elements to define an asymmetric resonant cavity;
- a planar wave guide formed in the substrate below the upper surface carrying the chromophore or chromophores, to capture a portion of the light emitted by the chromophore or chromophores into the substrate and/or to supply excitation light;
- a configuration of the upper surface of the substrate, formed as wells with a reflective bottom and filled with a material with a suitable index each receiving a chromophoric element;
- planar resonators formed in the upper surface of the substrate;

and in that the means enhancing the quantity of light emitted by the chromophoric element or elements comprise at least one of the means belonging to said group.

The device of the invention thus aims to modify the optical environment of each chromophoric element such that a large proportion of the light it emits is

collected. The yield is improved, along with the sensitivity of molecule detection tests.

In addition, for certain particular applications requiring excitation light for the chromophoric elements, the invention allows the excitation intensity to be reduced.

In some embodiments, this invention can also improve rejection of the excitation light collected in the detector, and as a result improves the signal-to-noise ratio (S/N).

In some embodiments, the invention can also reinforce optical excitation of the chromophores in addition to reinforcing collection of the light emitted by said chromophores.

A number of embodiments can be envisaged, depending on whether the modification to the environment of the chromophore relies on geometrical optics and/or wave optics effect.

In the field of geometrical optics, for example, where propagation of emitted and/or incident radiation (excitation radiation) is governed by the laws of geometrical optics, there may be provided:

- a first, "far" mirror integral with (or formed in) a substrate at a distance (d) from the chromophores, the distance being large with respect to the emission wavelength (λ), i.e., satisfying the criterion $d > n\lambda/2NA^2$ (where "NA" is the numerical aperture of the collecting lens and "n" is the index of the medium between the mirror and the chromophores), and intended to reflect the light emitted by the chromophoric elements towards the selected region in space. The first far mirror can be produced in the form of a stack of dielectric layers;

- microlenses each associated with a chromophoric element, and which can function in transmission or

in reflection, i.e., by focusing forwards or
backwards, as is the case with a Fresnel mirror;

o small wells, filled with a material with a
suitable index (preferably high) and the bottom of
which is reflective, and each capable of receiving
at least one chromophoric element onto this
filling material;

o diffraction means functioning in transmission or
in reflection and placed at a selected distance
from the chromophoric elements;

o if the chromophores emit into a planar waveguide
(the chromophores are not necessarily placed in
this guide), channels defining a blazed grating to
collect the light emitted by the chromophoric
elements in the direction of the support means and
to guide this light towards the collecting device;

o parallel linear structures produced in the upper
forward face of a substrate in the form of
channels (or grooves) or ribs, which may be U, V
or parabolic in shape, filled with a material with
a suitable index, with a reflective base, capable
of receiving at least one chromophoric element on
this filling material (between them, at their tops
or at their centers);

o a planar waveguide for guiding chromophore
excitation light by minimizing diffusion of this
light into the neighboring media;

o in the absence of a collection waveguide, it is
possible to provide a matrix of charge coupled
detecting (CCD) devices associated with the rear
face of the substrate, at least some of the
detecting elements being electronically addressed
in correspondence with at least one chromophoric
element. In this case, between the matrix and the
substrate, a layer forming an interference mirror
is advantageously provided, arranged so as to
reject chromophoric element excitation light. It

is also possible to couple the pixels from a CCD detector either to sites carrying active or empty chromophoric elements or non active chromophoric elements, or to sites having a reference activity

5 signal, to obtain, by differential measurement of the pixels from the CCD detector, the signal of the active chromophore with an increased S/N ratio by subtracting the background noise and stray excitation signal.

10 In the wave optics field, for example, where interference phenomena are employed to reinforce or reduce optical waves, it is possible to have:

○ a first reflector (or mirror) known as the "near" mirror analogous to that for geometrical optics

15 but placed at a distance (d) from the chromophores so that said reflector provides an interference effect to amplify collection of the light emitted into the support ($\underline{d}$ satisfies the relationship $d < n\lambda/2NA^2$), and coupled to the excitation waves

20 arriving at the chromophores with a non zero angle of incidence with respect to the normal to the support. This angle of incidence also reinforces excitation when double resonance exists, i.e., coincidence between the antinodes in the field for

25 the two excitation wavelengths (λ_{exc}) and emission wavelengths (λ_{em}). Detection remains centered on the normal to the support;

● this first mirror, placed below the chromophoric elements, can be combined with a second semi-

30 reflective mirror placed above these chromophoric elements, substantially parallel to the first mirror and at a selected distance from it to define an asymmetric resonant cavity, in particular of the Fabry-Pérot type, delivering the

35 light collected through the second mirror. In a variation, the second mirror or exit mirror can be below and the most reflective mirror at the

emitted wavelength can be above, this latter mirror possibly being substantially transparent at the excitation wavelength. In general, in the two cases, a double resonance condition analogous to that of the near mirror can be satisfied, taking distance d to be that which separates the chromophores from the most reflective mirror. The second mirror can be deposited directly either on the substrate or on the entrance face of an optical collector, or it may be constituted by the diopter of an existing entrance face;

- a planar waveguide capturing a large fraction of the light from the chromophores emitted into the support, and preferably provided with the means mentioned above for channeling the captured light towards the collecting device;

- a planar resonator formed in the substrate around each chromophoric element, intended to store the electromagnetic energy from the field it induces and arranged so that the associated chromophoric element is substantially positioned on an antinode in the electromagnetic field. It may comprise concentric circular channels or grooves (at least two) at the center of which is placed a chromophoric element. It may also comprise rectangular or parallel channels or grooves defining a lamellar grating;

- a two-dimensional (2D) or three-dimensional (3D) array of holes or columns defining a photonic crystal, preferably of the photon band gap type to control light propagation in its region, which may go so far as to forbid it;

- means for concentrating light that cause localized resonances by local reinforcement of the electromagnetic field induced by the presence of nanometric structures (for example islets) which may or may not be regular, or nanometric holes,

produced in selected metals, in particular silver
(Ag). These nanometric islets or holes are
capable of enhancing emission and also excitation
such as in surface enhanced Raman scattering

5 (SERS);

- means for concentrating light comprising an
asymmetric resonant cavity, in particular of the
Fabry-Pérot type, integrated into the substrate
below the upper layer, this layer being at least

10 partially permeable, vertically and/or laterally,
to allow the chromophoric elements to migrate
towards selected sites relative to the resonant
cavity. This cavity is preferably defined by
first and second mirrors, and the upper layer is

15 preferably produced in a porous material, for
example silica gel. Further, the upper layer can
comprise holes at selected locations to encourage
migration of the chromophoric elements towards the
sites.

20 Certain of the embodiments described above can be
taken in combination, to further improve light
collection.

The invention concerns all fields in which the
device described above can be used, in particular in

25 biology, biochemistry and chemistry.

More particularly, it concerns the use of a device
of the type described above and the use of biochips
intended to allow the detection of pairs of target and
probe oligonucleotides, such as DNA strands, detecting an

30 interaction between target polypeptide sequences or
proteins, labeled with a molecule that is capable of
directly or indirectly emitting chromophoric or
chromogenic signals, in particular luminescent,
fluorescent or colorimetric signals, and probe

35 polypeptide sequences or proteins, and detecting the
interaction between probe chemical molecules and target
chemical molecules.

.Other characteristics and advantages of the invention will become apparent from the following detailed description and the accompanying drawings, in which:

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- Figure 1 is a highly diagrammatic cross sectional view of a device in accordance with the invention, with a "far" mirror formed solely from dielectric layers, or hybrids of dielectric and metallic layers;

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- Figures 2A and 2B are highly diagrammatic cross sectional views of devices of the invention with mirrored wells;

- Figure 3A is a highly diagrammatic cross sectional view of a device in accordance with the invention,

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with a "near" mirror coupled to a non zero angle of incidence excitation; the curves of Figures 3B and 3C respectively illustrate graphs of the luminescence intensity ($|E_{fluo}(Z)|^2$) and excitation intensity ($|E_{exc}(Z)|^2$) as a function of the side

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(or thickness) $\underline{z}$;

- Figures 4A and 4B are highly diagrammatic cross sectional views of devices in accordance with the invention with a resonant cavity;

- Figure 5 is a highly diagrammatic cross sectional

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view of a device in accordance with the invention, with a planar resonator with grooves;

- Figure 6 is a highly diagrammatic cross sectional view of a device in accordance with the invention with a planar resonator and a waveguide;

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- Figure 7 is a highly diagrammatic cross sectional view of a device in accordance with the invention with a groove structure coupled with CCD type detection elements;

- Figures 8 to 14 are highly diagrammatic cross

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sectional views of different variations of the device in accordance with the invention;

⊙ Figure 15 is a highly diagrammatic cross sectional view of the top of the device of Figure 14.

The accompanying drawings illustrate definitive embodiments. As a result, they not only serve to describe the invention, but if necessary they also contribute to its definition.

In the following description, reference will be made to devices for supporting chromophoric elements comprising at least one support on which multiple zones are produced to receive probes which are to be coupled to targets. In the described application, the targets and probes are oligonucleotides such as DNA strands. However, the invention is not limited to that application; the targets and probes could be proteins or polypeptide sequences, or more generally any type of chemical, biochemical or biological molecule.

The device of the invention is intended to recover at least a fraction of the light photons emitted by chromophoric elements into a part of the region surrounding them, so that they are delivered to a selected region in space in which a collecting device is disposed, such as the objective of a microscope coupled to a photodetector, for example a photomultiplier or a matrix of photodetecting elements, for example of the CCD (charge coupled device) type.

To recover photons, the support device comprises means for concentrating light which can be classified into two different categories depending on whether they function in accordance with the laws of wave optics or of geometrical optics.

Reference should firstly be made to Figure 1 to describe an embodiment of the device of the invention functioning in accordance with the laws of geometrical optics.

The device comprises a support 1 formed from a substrate 2 in which a reflective mirror 3 is formed at a distance (d) from its upper face 24. The mirror can be

formed by depositing a layer that is highly reflective at the working wavelengths (emission wavelengths of the chromophores). In a variation and as illustrated, the mirror is constituted by a multiplicity of dielectric

5   layers 3. The substrate 2 and the dielectric layers 3 can be produced from semiconductor materials or from oxides or glasses. As an example, for an emission wavelength (λ_{emis}) of the order of 600 nanometers (nm), layers of SiO_2 can be selected (with a refractive index

10   n1 = 1.5) and with a thickness e1 = $\lambda/4n1$ = 100 nm, and layers of TiO_2 (with a refractive index n2 = 2.5) with thickness e2 = $\lambda/4n2$ = 60 nm. In this example, 2 to 10 pairs of layers are required, with an SiO_2 layer as the first layer.

15   However, other materials can be envisaged, such as nitrides (Si_3N_4) or organic or organometallic polymers, the polymers possibly being either amorphous (isotropic material) or "orientated" and birefringent. More details regarding the particularly advantageous reflective

20   properties of birefringent polymer layers, in particular when using two polarizations, can be found by referring to the article by Weber et al., in Science, vol. 287, p. 2451, March 31st, 2000.

In the illustrated embodiment, the mirror is "far"

25   so as to function in accordance with the laws of geometrical optics. The term "far mirror" means any type of reflective structure functioning, because of its distance from the chromophores, in accordance with the laws of geometrical optics and not in accordance with the

30   laws of wave optics (interference phenomena). In other words, the mirror is a distance d from chromophores 5, distance $\underline{d}$ satisfying the criterion: $d > n\lambda/2NA^2$; where "NA" is the numerical aperture of the objective collecting the light emitted by the chromophores at a

35   wavelength (λ), and "n" is the index of the medium between the mirror and the chromophores.

Such a "far" mirror, which is reflective, can multiply by about two the number of photons collected substantially around the direction N normal to the upper face 24 of support 1, the angle of incidence of the

5 excitation light on face 24 being any angle.

Once produced, the upper face 24 of support 1 first receives the different probes in the selected locations. The probes are fixed onto support 1 using addressing techniques that are known to the skilled person, which

10 depends on the nature of the probes and on the upper surface 24 of support 1. As an example, it is possible to use a chemical addressing technique, or electrochemical addressing, or a technique similar to the inkjet technique employed in printers.

15 Supports 1 equipped with their probes are then brought into communication with the targets, for example by immersion, so that certain targets interact with certain probes. The term "interaction" as used here means any type of coupling or hybridization or pairing

20 that can form target/probe pairs in a relatively durable manner.

As an example, the targets are DNA strands provided with luminescent markers. The term "luminescence" as used here means any type of light emission, induced or

25 spontaneous, such as photoluminescence, fluorescence or phosphorescence. In certain applications for which it is not possible to fix luminescent markers onto the target or the probe, luminescence is locally ensured by structures onto which the target/probe pairs are fixed.

30 This is particularly the case in certain semiconductor structures such as quantum wires and boxes (see the document by Bruchez et al., Science 281, p. 2013, September 25th 1998), or colorants whereby the pair fixed thereon acts as a luminescence destroyer or amplifier.

35 In this case, the substrate (mono or multi-layer) can be produced from semiconductive materials.

In this invention, therefore, the term "chromophoric element" means any element that is capable of directly or indirectly emitting a chromophoric signal or a chromogenic signal, following excitation by a suitable

5   light or after enzymatic transformation of a chromogenic substrate. It can thus be either a target/probe pair integral with the upper surface 24 of support 1 and which can spontaneously emit or be induced to emit light photons, or a three-dimensional structure which, once

10   associated with a target/probe pair, emits light spontaneously or in an induced manner, or an element that is integral with the substrate and which, once associated with a target/probe pair, changes its behavior emitting much more or much less than in the absence of the pair,

15   whether the emission is spontaneous or induced.

Thus, the invention aims to encourage collection of photons emitted by chromophores, and in particular all or a portion of the light that would be emitted in the direction of support 1. It should be remembered here

20   that about 70% of the light emitted by the chromophores is lost in the substrate and the remaining 30% is not necessarily captured by the collecting device, because of limitations in the collection aperture.

In a variation, support 1 of the device can comprise

25   microlenses that may or may not be structured and located substantially below each chromophore 5, functioning in transmission or in reflection (micromirror), i.e., by focusing forwards or rearwards, as is the case for Fresnel mirrors in particular. It is also possible to

30   use diffractive devices placed at distances that are sufficiently large compared with the wavelength of the emitted light. Under these conditions, the diffraction means function in accordance with the laws of geometrical optics, and their characteristics (angles of incidence

35   and emergence) are fixed by the laws of gratings.

One particular grating embodiment consists of redirecting the light using a synthesized holographic

grating (i.e., resulting from calculating an optical transfer function using methods known to the skilled person). More details regarding the ability of these holographic gratings to redirect waves guided into a

5   plane or outside a plane can be obtained by referring to Martinsson et al., IEEE Photon Tech. Letters 11, 503, 1999, and Larsson et al., SPIE Proc. 3626, 1999.

Reference should now be made to Figures 2A and 2B to describe a particularly advantageous further embodiment.

10   In this embodiment, small wells 19 (or three-dimensional reliefs) are formed in the upper portion 10 of support 1. These reliefs can be produced using different techniques for defining patterns (such as lithography and serigraphy, e-beam exposure), followed by techniques for

15   forming reliefs (such as chemical or dry engraving, using plasma, ion beam, plasma and/or reactive ion beam), and also directly by embossing.

After depositing a reflective layer 20 (or a multilayer assembly with reflective properties at the

20   wavelengths of interest) on the bottom of each well 19, it is filled with a filling material 18 with an index n2, preferably a high index, then chromophore 5 is placed on the upper surface of the filling material 18, substantially in the focal point (center) of the well 19.

25   A plurality (at least two) of chromophores can optionally be placed above the same well.

The reflective nature of the three-dimensional structures is produced by metallic or dielectric deposits. The wells are filled by any method suitable

30   for the desired material, preferably a dielectric method. Flat filling can be envisaged, such as in the case of an epoxy resin that solidifies flat, or by flattening in the case of a convex deposit, or without intervention, for example in the case of a concave dielectric.

35   Preferably, as shown in Figure 2B, wells 19 are parabolas of revolution, and the geometric focal point is located at the intended location for the chromophore (on

filling material 18), to permit idealized collection of a maximum of the emitted light. Emission from a chromophore placed a few nanometers (nm) from a n1/n2 interface sends the light mainly into a cone with an

5 angle spread between a critical angle $\theta c = sin^{-1}(n1/n2)$ and $\theta c + 10°$ or $20°$. More details regarding this mechanism can be obtained from the articles by Lukosz W. and Kunz, R. E., J. Opt. Soc. Am. Vol. 67, pp. 1615-1619 (1977), Lukosz W., J. Opt. Soc. Am. Vol 69, pp. 1495-1503

10 (1979), and Lukosz W., Phys. Rev. B. vol. 22, pp. 3030-3038 (1980). The well can also be an ellipse of revolution, to form an upstream real image of the chromophore located at the downstream focal point. Facetted wells of revolution can also be envisaged, the

15 facetting being n-dimensional, where n is 1 or more. It is also possible to produce wells with a three-dimensional facetting (n = 3), when the material lends itself to chemical engraving revealing preferred crystal planes.

20 It is also possible to concentrate the emitted light with simple grooves, for example in the form of a U or a V, or parabolic or even elliptical.

In the embodiments described above, it is also possible to improve rejection of the excitation light by

25 omitting to render reflective the regions of the wells illuminated by particular angles θ (including three dimensions). As an example, for a beam in a cone of revolution with an angle θ and a focal point located at the position of the chromophore, the reflective treatment

30 must be omitted over a ring 21, over a parabola of revolution, and if possible this region 2 must be rendered non reflective, non diffusing, but rather, absorbing, for example. Under these conditions, it is possible to obtain strong rejection combined with a high

35 signal by rendering reflective only the projection of this angular region onto the well, by fixing an angle of excitation that is not in this angular window.

Since the absolute quantity of light emitted spontaneously or in an induced manner by chromophores 5 increases with the index n2 of the filling material 18 because of the higher probability of a radiative channel,
5   which is proportional to a power of n2, materials with a high index n2 are preferably selected.

To detect channeled photons, the use of electronically addressed matrices of photodetecting elements is particularly advantageous, such as charge
10 coupled devices or devices formed using metal-oxide semiconductor (MOS) technology, being the cheapest currently available matrix detectors.

Preferably, these detectors are used to directly form support 1 or substrate 2, which prevents any loss of
15 light and retains radiation inclined beyond the critical angle of the support-air interface. With a detector separated from the support by a medium with a low index, such as air, these rays will be totally reflected and will not reach the detector.

20 CCD matrices can be produced on a silicon substrate, for example, so that they function at visible wavelengths, in the near infrared, or even in the near ultraviolet. These matrices are resistant to certain heat treatments, fluid or other, necessary in the
25 production of the device of the invention. Further, such photodetectors comprise photosensitive elements (or pixels) (as shown in the example of Figure 7) the dimensions of which can be in the range from 5 micrometers (μm) to 50 μm and which, as a result, enable
30 very high probe densities to be employed on that support.

When luminescence is induced, it may prove necessary to dispose of the photoexcitation light, which may saturate (or blind) or even destroy the photodetection elements. Two solutions can be envisaged. A first
35 solution is suitable for cases in which luminescence decreases more slowly than the period between two acquisitions. In these cases, it is possible to carry

out pulsed rapid photo-excitation to "empty" the detection pixels of the CCD matrix before acquisition. A second solution consists in adding mirrors, for example interference mirrors, or mirrors of another type (colored

5   filters, for example) or a combination of mirrors of different types, strong rejectors of excitation radiation (with a typical value of less than 10^{-4}, if possible less than 10^{-5}), while providing a transmission window for the radiation from chromophores 5. Clearly, these two

10   solutions can be combined together to further improve the collection results.

This means that microlenses or a mirror can be dispensed with, while retaining the possibility of carrying out complementary functions, of the wavelength

15   filtering type, at the collecting device.

The signal-to-noise (S/N) ratio can also be improved using a differential method of the type described below. A first detection pixel is placed facing each chromophore, and one or more other detection pixels are

20   placed to the side of this first pixel to collect the remainder of the emitted light, which has not been concentrated by the means of the invention, or any stray light. A differential measurement of the signal received by the pixel associated with the chromophore and the

25   signal received by the neighboring pixel or pixels produces a better S/N ratio and can dispense with various sources of noises and stray signals.

We now refer to Figures 3A to 3C to describe a further embodiment of a device of the invention,

30   functioning in accordance with the laws of wave optics.

More precisely, in this embodiment we create double resonance to reinforce the collected light and excitation light. The first resonance concerns the emission wavelength, while the second resonance concerns the

35   excitation wavelength. This double resonance is produced by determining the coincidence of the antinodes in the

electric field for the two wavelengths, excitation and emission.

A support 1 is used of the type described above with reference to Figure 1, i.e., comprising a reflective mirror 3 formed from dielectric or metallic layers. However, in contrast to the example of Figure 1, where the mirror is a far mirror, in this embodiment the mirror is a "near" mirror. In other words, it is placed at a distance (d) from the chromophores that satisfies the criterion: $d < n\lambda/2NA^2$.

Under these conditions, an interference effect is produced that can enhance the collected energy by a factor of four. More details regarding this mechanism can be obtained from the article by Benisty et al., IEEE J. Quantum Electron, vol. 34, 1612, 1998.

This near mirror condition is combined with a condition regarding the angle of incidence θ of the excitation light with respect to the normal N to the upper surface of support 1 (concentrated light is collected substantially perpendicular to the upper surface of support 1).

The spacing <u>a</u> of the field antinodes at wavelength λ in a medium with an index <u>n</u> illuminated by a wave the incidence of which in an external medium, with an index n_{ext}, is θ, is given by:

$a = (\lambda/2n)/\cos(\arcsin((n_{ext}/n)*\sin(\theta)))$.

a_{exc} is the spacing of the antinodes for an excitation at an angle θ, and a_{fluo} is the spacing of the antinodes for fluorescence detected at θ_{fluo} (generally at about 0 degrees, but this angle can be offset to the middle of the collection window of the lens, i.e. : $\sin(\theta_{fluo}) = NA/2$.

The choice of excitation angle θ (for a conical beam) allows the spatial position of the antinode for the excitation light (λ_{exc}) to be adjusted and to achieve adequate spatial coincidence with the antinode of the emitted light (λ_{fluo}).

More precisely, θ must be found by satisfying the following condition:

$$(p + \tfrac{1}{2}) * a_{exc} = (m + \tfrac{1}{2}) * a_{fluo}$$

where $\underline{p}$ and $\underline{m}$ are the smallest possible whole

5   numbers. This is achieved by starting from a convenient low value for $\underline{m}$ of less than 20, and trying values of $\underline{p}$ that are lower than or equal to $p_0 = m * (\lambda_{fluo}/\lambda_{exc})$, or a little higher if the penetration depths of the mirror vary rapidly with the wavelength in the region under

10   consideration. Values of θ are found to increase as $\underline{p}$ decreases; we stop at the pair (m, p) that is the most convenient and the smallest, taking into account the other constraints on support manufacture.

For better adjustment, the penetration depths a_{pen}

15   into the mirrors must also be taken into account, as the mirrors are not ideal mirrors. These depths are only a small fraction of λ for metals, but are given by $(\lambda/2n_{mean}) * (n_{mean}/2\Delta n))$ for a multilayer Bragg mirror type stack, n_{mean} being the mean of the indices of the two

20   layers and Δn being their difference in index.

The exact condition to be satisfied to find θ is then:

$$(p + \tfrac{1}{2}) * a_{exc} + a_{pen}(\lambda_{exc}) = (m + \tfrac{1}{2}) * a_{fluo} + a_{pen}(\lambda_{fluo})$$

It should be noted that in this case, optimization

25   of the thicknesses and compositions of the reinforcing absorption layers does not in practice depend on the numerical apertures considered.

It is also possible to envisage that an assembly of layers could be anti-guiding as regards the plane of the

30   chromophores 5, i.e., that the tendency of the modes that propagate in this assembly to capture the light emission is lower than a substrate with the same mean index as these layers. Photons that are not emitted in this assembly of layers are then found substantially in the

35   collection cone, especially when this cone is quite large.

Reference should now be made to Figure 4A to describe a further embodiment of a device of the invention, functioning in accordance with the laws of wave optics.

In this embodiment, a support 1 is provided of the type described above with reference to Figure 3A, i.e., a substrate 2 comprising a near mirror, reflective, formed from dielectric layers 3, for example, and a second mirror 7, semi-reflective, placed above the chromophores at a set distance (d) from the upper surface 4 of the first mirror. The distance $\underline{d}$ between this upper surface 4 of the first mirror and the lower surface 8 of the second mirror 7, facing it, is selected so that the two mirrors define an asymmetric resonant cavity of the Fabry-Pérot type.

The distance (d1) between the first mirror and the chromophores is selected to ensure the presence of a field antinode at chromophores 5, and is thus substantially d1 = $(m + \frac{1}{2}) * \lambda_{fluo} + a_{pen}(\lambda_{fluo})$. The excitation angle θ can be selected in the same manner as indicated above for a simple mirror, and the most favorable of the values of θ are selected taking the properties of the second mirror into account.

The second semi-reflective mirror 7 allows photons emitted by chromophores 5 placed on the upper surface 4 of first mirror 3 to traverse it, and as a result to be detected by a collecting device (placed above the second mirror 7). Such a second semi-reflective mirror 7 can advantageously be formed by the lower face of an objective of the collector. It may be that face directly, or a coating deposited thereon. Since adjustment of the resonance wavelength with respect to the emission wavelength (tuning) must be quite accurate (of the order of about 1%), an adjuster can be provided on one of the elements of the device (for example the support or an external mirror) of the piezoelectric type controlled by an optical in situ measurement of

resonance. Further, this adjuster can be envisaged to be coupled to the collecting device, or mounted so as to allow the surface of support 1 to be scanned.

In a variation, the second semi-reflective mirror 7
5 can be formed on the lower face of a coverslip of the type used for microscopic observations. Because of the very small dimensions of resonant Fabry-Pérot type cavities, typically $\lambda/2$, the coverslip can be placed over the chromophores 5, interposing a fluid (constituted, for
10 example, by the biological sample itself or by a wetting liquid), and the cavity thickness is then controlled by controlling the pressure applied to the coverslip, which can optionally be coupled to an automatic optical control.

15 In a further embodiment of the invention, shown in Figure 4B, the resonant cavity is made all of a piece, the upper layer and the upper mirror 7 being integral with the substrate 2, and the sites for positioning the chromophores 5 being located inside the structure at
20 optically suitable positions, given above, relative to the resonant cavity. The resonant cavity is preferably of the asymmetric Fabry-Pérot type, and more generally delimited by two mirrors 3, 7 spaced apart by a distance (d) selected mainly as a function of the working
25 wavelength and the materials used. These two mirrors are preferably of the type described above, i.e., in the form of a multilayer dielectric structure.

Access to the internal sites by the chromophores 5 is achieved by any means rendering the upper layer 22 at
30 least partially permeable, vertically and/or laterally. As an example, porous materials such as silica gel can be used. These materials can also include holes to facilitate migration of the chromophores towards the internal sites.

35 Reference should now be made to Figure 5 to describe a further embodiment of a device of the invention, in

which the light concentrating means function in accordance with the rules of wave optics.

In this embodiment, support 1 is formed from a substrate 2, the supper surface 24 of which comprises a
5 multiplicity of holes 11 each intended to receive a target. The holes allow the chromophores to be placed in locations where the field of the resonator is a maximum. Their depth is between zero and the depth of the grooves of the resonator, described below. These holes are
10 preferably arranged in a regular manner. To collect the light emitted by chromophore 5 formed by interaction of a probe with the target lodged in a hole 11, a planar resonator 12 is formed around each hole 11.

Such a resonator can, for example, be constituted by
15 a series of concentric channels (or grooves) 13, opening at the upper surface 24 and with widths and depths selected to provide the properties of confinement and extraction of the light emitted at their center. More precisely, this resonator is arranged so that the
20 electric field has an antinode at the level of hole 11 containing chromophore 5 and so that it stores electromagnetic energy, the light being recovered at the channels (or grooves) 13 then transmitted to the portion located above the surface 4, following (approximately)
25 the arrows, to be captured by a suitable collecting device.

The grooves can be a few micrometers deep or can equal or exceed the radii. The groove width must be equal to about a fraction of the emitted wavelength,
30 typically of the order of 0.1 μm.

The size of the circular resonator is determined by the following condition:

radius = $(\lambda/2\pi n_{eff}) * z_{m,n}$

where $z_{m,n}$ is the nth zero of the Bessel function of
35 order m, $J_m(z)$. This is meant for a resonator with a perfect lateral mirror; n_{eff} is the mode of the subjacent

planar guide if it exists, if not it is the index of the solid in which the resonator is defined.

For multilayer mirrors, the presence of an antinode at the edge of the resonator must be taken into account instead of a field node at the edge of a perfect mirror resonator. Thus, a quarter wavelength has substantially to be added:

$$radius = (\lambda/2\pi n_{eff}) * z_{m,n} + \lambda/(4n_{eff}).$$

Preferably, the groove pitch is substantially equal to a multiple of $(\lambda/2\pi n_{eff})$ or very slightly higher (5% to 20%). This choice is suitable for very thin multilayers, $\lambda/8$ to $\lambda/50$, the lower limit usually being fixed by technology. This groove width controls the penetration length of the wave into the circular "mirror".

Other types of planar resonator can be envisaged. Lamellar arrays can be produced with rectangular grooves or parallel grooves with dimensions similar to those mentioned above. A two-dimensional or three-dimensional photonic crystal can also be produced by forming holes or hollow column placed in selected locations in the substrate, at its upper portion 10. As an example, the holes can be round and form a triangular array, omitting a certain number of them to form the resonant cavity.

Such planar resonators (concentric grooves and photonic crystals) are described in the document "Optical and confinement properties of two-dimensional photonic crystals", J. Lightwave Techn., vol. 17, pp. 2063-2077, 1999, H. Benisty, C. Weisbuch, D. Labilloy, M. Rattier, C. J. M. Smith, T. F. Krauss, R. M. De La Rue, R. Houdré, U. Oesterle and D. Cassagne.

If their dimensions are selected for resonance with chromophore 5, these planar resonators have resonant modes that can remove a large proportion of the emission from this chromophore. These resonant modes are shown highly diagrammatically as the dotted lines in Figure 5. Further, these planar resonators ensure coherent diffraction, similar to Bragg type diffraction, which can

redirect the energy of the resonant mode towards the collecting device.

In a variation, a very small resonator can be used, typically $2\lambda/n$ or $3\lambda/n$. In this case, the resonator can be anti-resonant over the whole wavelength range of chromophore 5, which inhibits emission in the plane and encourages emission along the vertical, i.e., perpendicular to the upper surface 24. Coupling of the photodetecting elements then allows the light to be collected.

In the case of photonic crystal type structures, it is possible to use those known as photon band gap type crystals which have the property of forbidding propagation of light through them, forcing all of the electromagnetic energy of the light emitted by the chromophore 5 outside them, even when the chromophore 5 is located at a distance of several wavelengths (λ) from the interior of the photonic crystal.

When no longer resonant, i.e., when they have sizes or radii of more than about 10λ, the groove structure or photonic crystal type structure can be used to diffract the light guided from the chromophore. These structures tend towards geometrical optics and function well for a certain variety of incident wavelengths and no longer for one or more overriding modes. This configuration has the advantage of requiring less rigorous dimensional tolerances of the probes in the horizontal position, while in the case of a resonator, the size of the antinode in the electric field must typically be about $\lambda/2n$, i.e., less than about 200 nanometers.

Concentric grooves, lamellar gratings (rectangular, parallel or V shaped grooves or the like) and photonic crystals all have spectral/angular windows in which their behavior is not reflective and can be rendered extractive. Optionally, extraction can be carried out via a substrate, on the back of which microlenses (functioning in accordance with the laws of geometric

optics) can be placed to augment the quantity of light collected.

In a further embodiment of the invention, localized resonances are created of the type that occur in surface enhanced Raman scattering. These resonances result from local reinforcement of the electromagnetic field induced by the presence of nanometric structures, such as nanometric islets, or nanometric holes, formed from particular metals such as silver (Ag) or gold (Au). Local reinforcement primarily concerns emission, and also excitation. More precisely, it is possible to use resonances either to reinforce the excitation light (λ_{exc}), or to couple the light emitted by the chromophores (λ_{emis}) to collecting means. To this end, it is possible to deposit on a simple substrate, or a structured substrate as described above, irregular films of silver (or organized silver nanostructures) onto which probes are placed. More details of these nanometric structures or holes can be obtained by reference to articles by W. L. Barnes and V. A. Shubin et al., published in IEEE Journal of Lightwave Technology, vol. 17, p. 21-83, November 1999.

Reference should now be made to Figure 6 to describe a further embodiment of a device of the invention, in which the light concentration means also function in accordance with the laws of wave optics.

In this embodiment, a planar waveguide is provided below the portion of the support on which the chromophores 5 are fixed. To this end, for example, it is possible to deposit a high index layer 14 on the upper face of a substrate 2, the index of the high index layer also being higher than that of the upper layer 16 carrying chromophores 5. However, the structure can remain a guiding structure in the absence of the upper layer 16, as the skilled person will know.

More complex guiding structures can be envisaged to position the guided modes of the light emitted by the

chromophores in a particular manner, for example so that the field is as strong as possible at the surface. Such a structure can, for example, comprise a substrate 2 with a very low index (no upper layer 16) and a guiding layer

5   with an index and thickness selected at the limit of the mode cutoff condition. More details on this cutting condition can be obtained by reference to Chapter 8 in "Light Transmission Optics" (Van Nostrand, New York, 1972) by D. Marcuse.

10   Such guides can be monomodal or multimodal in type. In this latter case (multimodes), if the number of modes is really high, and as a result, the guide is very thick, then the structure will function in accordance with the laws of geometrical optics. Further, this type of

15   guiding structure can be very restricting or less restricting depending on whether there is a large or small difference in index between the surrounding layers. Further, these guiding structures can be optimized so as to capture a large fraction of the light, either in a

20   single mode, or in an overriding proportion of these modes if they are multimodal, to facilitate light extraction.

When the light concentration means comprise only a guiding structure 14, the guided modes are channeled to

25   the front of the structure. It is then possible to collect the light by coupling the ends of the waveguide to a suitable collecting device, such as fiber optics, or microscope objectives.

It is also possible to focus the collecting device

30   at different depths inside the guiding structure to isolate successive lines of chromophores 5. One example of this type of focusing is described in the document "Use of guided spontaneous emission of a semiconductor to probe the optical properties of two-dimensional photonic

35   crystals", Appl. Phys. Lett., vol. 71, pp. 738-740, 1997, D. Labilloy, H. Benisty, C. Weisbuch, T. F. Krauss, R. Houdré and U. Oesterle.

To allow collection of guided modes on a single side of support 1, groove or channeled structures can be provided around each chromophore and open into the guiding structure, to produce a species of blazed grating
5   that channels the emitted light to a service side of the guiding structure, where detection is made.

As illustrated in Figure 6, it is also possible to add to the guiding structure 14 a further structure forming a planar resonator, of the type described with
10   reference to Figure 5. As an example, it is possible to form concentric grooves (or channels) 15 around chromophore 5, to redirect the guided modes via the guiding structure 14 towards a zone located above said chromophores 5.

15   In a variation, it is possible to replace the grooves or channels 15 by a two-dimensional or three-dimensional photonic crystal constituted by holes or columns, as described above.

Reference should now be made to Figure 7 to describe
20   a further embodiment of a device according to the invention, also functioning in accordance with the laws of wave optics.

In this embodiment, the device of the invention comprises a substrate 2 the upper portion 10 of which is
25   formed with relief structures 6, with recesses or bosses. Preferably, these structures 6 are channels or ribs that are linear and parallel to each other, for example in the form of a U or a V (as in the example shown). The size of these structures is of the order of the working
30   wavelength. The probes can be placed either at the top of the structures (as shown) or at the bottom of the structures, i.e., in the space joining the lower portions of structures 6. It is also possible to associate metals with dielectric supports, for example by depositing
35   patterns of metal over all or some of the ribs or grooves.

As is known by the skilled person, at certain locations, in particular their top, such structures induce channeling effects on the light (see the arrows in Figure 7). As a result, by placing the photodetecting elements (or pixels) 17 below the relief structures 6 on which chromophores 5 are fixed, it is possible to harvest the photons emitted by the chromophores into the structures and channeled thereby. The light emitted in the direction substantially opposite to the direction of the channeled light can be collected by other photodetectors placed above chromophores 5. As indicated above, these photodetecting pixels can be CCD elements forming a detection matrix, for example.

Integration of a CCD photodetecting matrix 17 with a substrate 2 carrying chromophores 5 is shown more completely in Figure 8, which shows that substrate 2 comprises, below chromophores 5, reflective means 3 with a multilayered structure, and an absorbent layer 23 that is insensitive to angle of incidence and located between reflective layers 3 and photodetectors 17. The reflective layers 3 ensure a maximum excitation and emission field at the chromophores and prevent the excitation light from propagating by simple transmission and by diffusion towards the photodetecting matrix 17. The absorbent layer 23, as used for RGB filters in photography and in color CCD arrays, has an absorption band and thickness selected to reduce cross-color and/or enhance the signal-to-noise ratio at photodetectors 17. This produces a gain of 10 or 100 times as regards extinction of the excitation light, without greatly degrading collection of the fluorescence emitted by chromophores 5.

These improvements also apply to an embodiment of the invention where the CCD detector is not integral with substrate 2 carrying chromophores, but located at a sufficiently small distance for each chromophoric element to be facing sufficiently well defined pixels.

In the variation shown in Figure 9, chromophores 5 carried by substrate 2 are excited by a damped wave. To this end, substrate 2 comprises a guiding layer 25 analogous to the layer 14 in Figure 6, with an index that

5 is higher than that of the surrounding layers and wherein the upper face is very close to chromophores 5 on the scale of the wavelength of the damped wave of the guided wave. The guiding layer 25 is edge lit (after cleaving or cutting and polishing) or by means of a grating (for

10 example, a thin layer 26 of photosensitive resin on the guiding layer in a neutral zone of substrate 2, with a low thickness modulation of 1 to 100 nm, allowing light to be coupled by illumination using a laser with a suitable coupling angle determined by applying the law of

15 gratings. Regarding the remainder, the device of Figure 9 contains reflective layers 3 and absorbent layers 23 that have already been described with reference to Figure 8.

In a variation, chromophores with different colors are detected. The chromophores are illuminated by one or

20 are detected. The chromophores are illuminated by one or more excitation wavelengths λ_{exc1} and possibly λ_{exc2} and in response, emit two wavelengths, respectively λ_{fluo1}, λ_{fluo2}. In this case, it is possible to recover separately the signals for each color on pixels or close groups of

25 pixels all facing chromophores 5 using wavelength filters such as those described above, but individualized to each pixel or group of pixels. In addition to detecting two colors, this can use the advantages of differential treatment of the signals from the two colors. The

30 principle extends to several colors.

A further embodiment, shown diagrammatically in Figure 10, allows the detection of chromophores with different colors at each point 5 on substrate 2. The device is reminiscent of that of Figure 9 and

35 additionally comprises a second matrix 27 of CCD photodetectors 17 placed above the chromophores, interposing an objective 28 and a rejecting filter 29

between the chromophores and the second matrix 27. The chromophores 5 are illuminated by two excitation wavelengths λ_{exc1} and λ_{exc2} and in response, emit two wavelengths, respectively λ_{fluo1}, λ_{fluo2}. The absorbent

5   layer 23 of substrate 2 forms a spectral filter for rejecting excitation wavelengths and one wavelength λ_{fluo2} emitted by chromophores 5, by allowing the other wavelength λ_{fluo1} emitted by chromophores 5 to pass through. Filter 29 associated with the second matrix 27

10   stops the excitation wavelengths and the wavelength λ_{fluo1} emitted by chromophores 5, allowing the wavelength λ_{fluo2} to pass through. If necessary, an absorbent layer 23 of the type described above can be associated with filter 29.

15   A variety of light collecting devices are diagrammatically shown in Figures 11 to 16 by way of example only.

         The collecting device in Figure 11 comprises a CCD type photodetecting matrix 27 or the like receiving the

20   luminescence emitted by the chromophores carried by support 1, passing through a filter 29 for rejecting excitation light and stray luminescence emitted by support 1. Excitation of chromophores by a wavelength λ_{exc} takes place at a non zero angle of incidence with

25   respect to the normal to the support 1, by means of a dark field type coupler, routinely used in episcopy and metallography, placed between the support 1 and filter 29. It receives the excitation light from the side and returns it towards the support at angles of incidence

30   that are higher than the numerical aperture of objective 31. Objective 31 collects the luminescence emitted by chromophores in the direction perpendicular to support 1.

         In the variation shown in Figure 12, we see the photodetector matrix 27 and filter 29 of the device of

35   Figure 11, and support 1 is illuminated by a separator cube 32 associated with a mirror objective 33 of the Cassegrain type (Cassegrain-Schmitt) which encourages

detection at a non zero angle with respect to the normal to support 1, as shown by the arrows. Excitation passes via a central opening formed in the reflecting mirror of the objective and falls on support 1 with an almost

5   normal angle of incidence.

The collecting device of Figure 13 corresponds to that described above with reference to the resonant cavity of Figure 4A: in this device, the second mirror 7 is formed on the lower face of the entrance lens 34 of an

10 objective 35 of the collecting device. The optical path between mirrors 3 and 7 is a multiple of $\lambda_{fluo}/2$.

In the device of Figures 14 and 15, which corresponds to the embodiment of Figure 6, luminescence emitted by chromophores 5 into an upper guiding layer 25

15 of substrate 2 is recovered at one end by an objective shown at 36. Figure 15 shows the situation in which the guide points matching with the detector encourage selective detection of one of the chromophores on which focusing is better, compared with the others, in which

20 emission is not in the focal plane and is not concentrated in the detector (or in the pixel in the detector matrix), which allows discrimination between the chromophores located at different distances from the edge of the guide.

25